Journal of Medical Virology 51:332–337 (1997)

Impaired Interferon Induction of Human MxA Protein in Chronic Hepatitis B Virus Infection

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MxA protein is interferon inducible, and its role as an antiviral mediator is being studied in various viral diseases. Several cytokines, including type I interferons (α and β), interleukins 2 and 12, and granulocyte, macrophage, and granulocyte-macrophage colony-stimulating factors, were tested for their ability to induce human MxA protein synthesis in peripheral blood mononuclear cells from 15 chronic hepatitis B virus-infected patients and 6 healthy subjects as controls. Constitutive MxA expression was scarce in patients and controls but increased significantly in response to type I interferons. MxA responsiveness to interferon α was diminished significantly in chronic hepatitis B patients, compared with healthy donors ($P < 0.05$); this effect was more marked in patients with high viremia levels. Interleukins 2 and 12, and none of the colony-stimulating factors tested, induced low, but detectable, MxA protein levels. These results indicate that chronic infection by hepatitis B virus may impair activation of the immune cells and their capacity to respond to type I interferons. ***J. Med. Virol. 51:332–337, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: chronic hepatitis B virus infection; MxA protein; type I interferons; interleukins; colony-stimulating factors

INTRODUCTION

During the natural history of a viral infection, several cellular and molecular mechanisms are triggered to establish an antiviral state at the target cell. These mechanisms are of two types: the induction of a variety of cytokines, which leads to the establishment of an appropriate immune response; and the activation of cellular genes whose expression can interfere with one or more steps in the viral replicative cycle. Several products of these cellular genes have been identified, including the 2′,5′-oligoadenylate synthetase (2-5A), the RNA-dependent protein kinase, and the Mx proteins [Staeheli, 1990].

The Mx genes belong to a family of type I interferon (IFN)-induced proteins which were described first in the murine system as a gene locus containing two linked genes, called Mx1 and Mx2, of which only Mx1 is synthesized, conferring resistance to the influenza virus infection [Staeheli, 1990]. Similarly, two proteins, termed MxA and MxB, were found in the human system [Horisberger and Hochkeppel, 1987], and their cDNAs were cloned [Aebi et al., 1989; Horisberger et al., 1990]. The human MxA protein, unlike the mouse Mx1, covers a broad range, and inhibits several viruses, such as measles virus [Schnorr et al., 1993], vesicular stomatitis virus [Staeheli and Pavlovic, 1991], Puumala virus [Temonen et al., 1995], tick-borne Thogoto virus [Frese et al., 1995], and the influenza virus [Pavlovic et al., 1992].

Jackschies et al. [1994] reported recently that peripheral blood mononuclear cells (PBMC) from patients infected by hepatitis A virus (HAV) in the acute phase show a strong, but transient, expression of MxA, while patients infected by hepatitis B (HBV) or C virus (HCV), also in the acute phase, produce low levels of MxA protein. The expression of the MxA protein has not been investigated during the chronic phase of hepatitis B. We examined the patterns of MxA expression in response to a variety of cytokines in PBMC obtained from patients with a chronic HBV infection, compared with those from healthy donors.

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Accepted 6 November 1996

TABLE I. Characteristics of Patients

Patient no.	Age/sex	Time of known HBsAg carriage (years)	Route of infection	ALT/AST (IU/ml)	Serum markers	HBV-DNA (dot-blot/PCR)	Histology
1	43/M	7	Unknown	27/20	Anti-HBe+	–/+	Moderate activity
2	45/M	10	Vertical transmission	79/44	Anti-HBe+	–/+	NA[a]
3	42/F	6	Unknown	29/19	Anti-HBe+	–/+	NA
4	60/M	5	Transfusion	37/35	Anti-HBe+	–/+	Moderate activity
5	25/M	7	Unknown	34/59	Anti-HBe+	–/+	Moderate activity
6	50/M	10	Unknown	57/86	Anti-HBe+	–/+	Moderate activity
7	48/F	3	Unknown	31/48	Anti-HBe+	–/+	Moderate activity
8	18/M	4	Unknown	68/35	HBeAg+	+/+	Minimal activity
9	44/M	3	Unknown	86/135	HBeAg+	–/+	NA
10	34/M	5	Unknown	842/421	HBeAg+	+/+	Moderate activity
11	61/F	7	Unknown	593/306	HBeAg+	–/+	Moderate activity
12	21/M	13	Vertical transmission	38/82	HBeAg+	+/+	Mild activity
13	36/M	7	Unknown	42/48	HBeAg+	+/+	Moderate activity
14	41/M	7	Unknown	62/109	HBeAg+	+/+	Moderate activity
15	26/F	22	Transfusion	31/48	HBeAg+	+/+	Mild activity

[a]NA: not available.

PATIENTS AND METHODS

Patients

Fifteen patients with chronic HBV infection were examined for expression of the MxA protein in PBMC. Of these, seven were anti-HBe positive, and eight were HBeAg positive. Patient characteristics are summarized in Table I. Patients who had HBV DNA in serum by dot-blot hybridization are referred to as having high levels of HBV replication, and patients with HBV DNA detectable only by the polymerase chain reaction (PCR) are referred to as having low levels of HBV replication. A histological diagnosis was available in 12 cases and was established according to Desmet et al. [1994]. No patient had superimposed hepatitis C or D or human immunodeficiency virus type 1 infection; other causes of liver disease were excluded. None of the patients had received antiviral or immunosuppressive therapy. As controls, PBMC from six healthy donors with normal serum alanine transaminase (ALT) levels, and without viral hepatitis markers, were also analyzed for expression of the MxA protein. All patients, as well as the healthy subjects, had comparable blood cell counts within the normal range. This study was conducted in accordance with the Declaration of Helsinki on human experimentation.

Methods

HBV serological markers (HBsAg, anti-HBc, HBeAg, and anti-HBe) were assayed by commercial radioimmunoassays (RIA; Abbott Laboratories, North Chicago, IL). HBV DNA was tested in serum by dot-blot hybridization [López-Alcorocho et al., 1994] and, whenever testing by this technique proved negative, the serum was examined by the PCR [López-Alcorocho et al., 1994].

PBMC Culture

PBMC were isolated from heparinized venous blood by Ficoll-Hypaque gradient sedimentation (Seromed, Biochrom KG, Berlin, Germany). Isolated PBMC were seeded onto a 96-well culture cluster (Costar, Cambridge, MA), in 0.2 ml of the RPMI-1640 medium supplemented with 10% of fetal calf serum, at a density of 2.0×10^5 cells per well. Cells were cultured at 37°C in a humid atmosphere with 5% CO_2 for 24 hr either unstimulated or stimulated with 10, 100, and 1,000 IU/ml of recombinant human IFN-α (rhIFN-α) 2a (F. Hoffmann-La Roche, Basel, Switzerland); rhIFN-α2b (Schering-Plough Corp., Kenilworth, NJ); lymphoblastoid IFN-αn1 (Wellcome Foundation Ltd., London); natural human IFN-β (Serona Laboratories, Madrid, Spain); interleukin (rhIL)-2 (Chiron-Cetus, Emeryville, CA); 10, 100, and 1,000 ng/ml of macrophage colony-stimulating factor (rhM-CSF); Chiron-Cetus); granulocyte-macrophage colony-stimulating factor (rhGM-CSF; Schering-Plough Corp.); 1, 10, and 100 ng/ml of rhIL-12 (R&D Systems, Minneapolis, MN); and granulocyte colony-stimulating factor (rhG-CSF; Amgen Inc., Thousand Oaks, CA). At the end of the culture period, the cells were centrifuged and were separated from wells with trypsin-EDTA and treated with lysing buffer (0.5% NP40, 25 mmol/L HEPES, 5 mmol/L $MgCl_2$, 10% glycerol, 1 mmol/L PMSF, 10 μmol/L leupeptin, 1 mmol/L benzamidine, and 1 mmol/L EDTA), as described by Martín et al., [1993].

Immunoblot Assay for Human MxA Protein

The immunoblot assay for human MxA protein has been described in detail elsewhere [Jakschies et al., 1990]. Briefly, aliquots of lysed PBMC were boiled for 5 min in 50 mM TRIS-HCl, pH = 6.8, 3% sodium dodecyl sulfate (SDS), 3% glycerol, and 5% DTT and subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE). The gel was blotted to Immobilon-P (PVDF) membrane (Millipore, Bedford, CA). After blocking with non-fat dry milk, the membrane was incubated for 1 hr with a monoclonal antibody which recognizes the human MxA protein [Horisberger and Hochkeppel, 1987], diluted 1:3,000 in phosphate buffered saline-

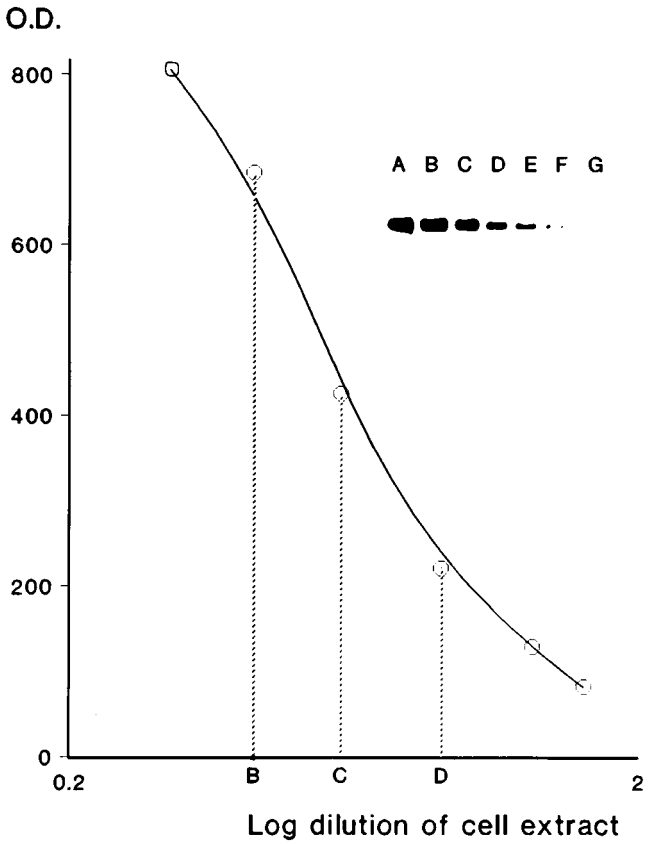

Fig. 1. Standardization of MxA immunoblot assay. 2.0×10^6 PBMC from a healthy donor were cultured for 24 hr in 1,000 IU/ml rhIFNα-2a and lysed. Serial dilutions were assayed, as described; immunoblot results are shown in the upper right corner. Optical density of the bands was plotted. The curve stretch between dilutions B, C, and D (corresponding to 7.5×10^4, 5.0×10^4 and 2.5×10^4 cells, respectively) was almost linear, and these dilutions were subsequently chosen for quantification of the MxA protein.

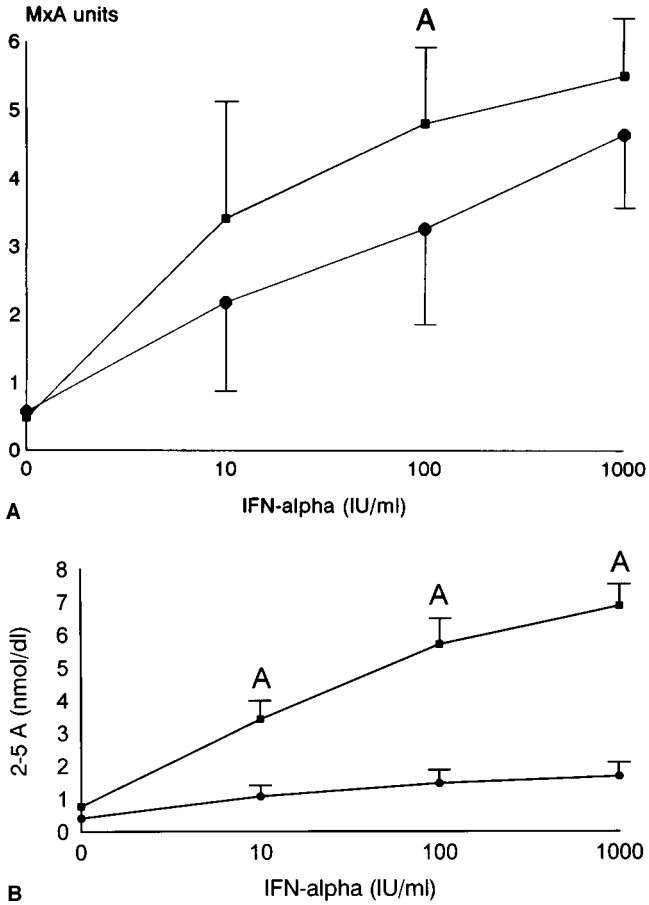

Fig. 2. The MxA protein and 2-5A synthetase in PBMC from chronic hepatitis B patients and healthy donors. Levels of MxA protein **(A)** and 2-5A synthetase activity **(B)** in PBMC from healthy donors (squares (n = 6) vs. chronic hepatitis B-infected patients (circles) (n = 15); bars represent the mean ± standard deviation. A: $P < 0.05$ by Student's t test.

0.1% tween 20 (v/v). After washing, a peroxidase-conjugated rabbit anti-mouse antibody (Dakopatts A/S, Denmark) diluted 1:1,000 was placed on the PVDF for 30 min. Extensive washing was carried out, and the membrane was revealed by autoradiography with the detection reagent of the enhanced chemiluminescence (ECL Amersham, Buckinghamshire, UK). The absorption of the human MxA protein band was recorded with a laser densitometer (Molecular Dynamics), and the area under the curve was integrated. For standardization of MxA detection and quantification, 2.0×10^6 PBMC from a healthy donor were cultured in the presence of 1,000 IU/ml rhIFNα-2a for 24 hr, and serial dilutions of the cellular extracts (ranging from 1.0×10^5 to 5.0×10^3 cells) were assayed as described above. As shown in Figure 1, the curve stretch between dilutions corresponding to the 7.5×10^4 and 2.5×10^4 cells was almost linear. Therefore, these three dilutions were included in every electrophoresis run and used to determine the amount of MxA protein. Five units of MxA protein were defined as the concentration present in a cell extract dilution of the 5.0×10^4 cells.

2-5A Activity

The activity of the enzyme 2-5A was measured in cell lysates from PBMC, obtained as described above by a sensitive RIA (Eiken Chemical Co., Tokyo, Japan), with an assay range between 10 and 810 pmol/dl and an intrassay coefficient of variation of less than 15%.

Statistical Methods

Data were analyzed using the Student's t test for mean comparison, one-way analysis of variance with two tails, and Pearson's correlation coefficient.

RESULTS

Unstimulated PBMC from patients with chronic HBV infection showed a very low, or undetectable, constitutive expression of the MxA protein (basal levels), and MxA expression did not differ from those levels found in PBMC from healthy donors (Fig. 2A). When mononuclear cells were cultured in the presence of 10-fold increasing concentrations of IFN-α, PBMC from healthy donors showed a strong, dose-related, and significant ($P < 0.001$) induction of MxA expression (Fig.

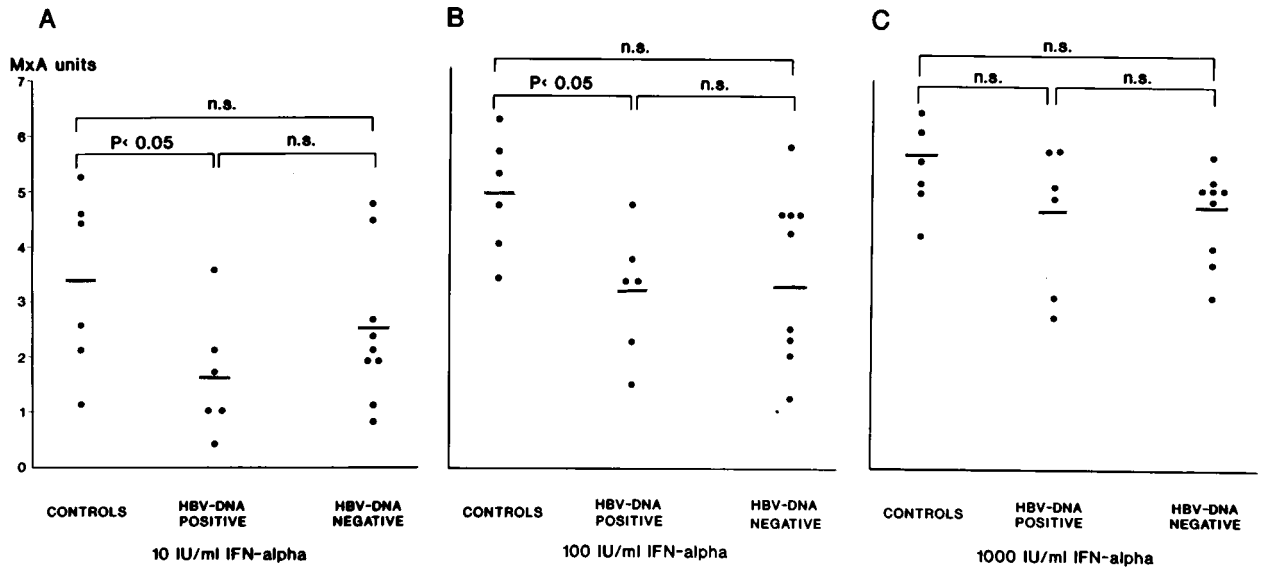

Fig. 3. Relation between individual levels of the MxA protein and HBV viremia. PBMC from patients and healthy donors were cultured for 24 hr with IFN-α at a dose of 10 IU/ml **(A);** at a dose of 100 IU/ml **(B);** at a dose of 1,000 IU/ml **(C),** and analyzed for MxA expression by Western blot analysis. Horizontal bars represent the mean value. n.s., not significant.

2A). In PBMC from chronic HBV-infected patients, MxA expression in response to IFN-α was also significantly ($P < 0.001$) induced in a dose-dependent manner in all cases (Fig. 2A). However, MxA expression was induced in chronic HBV patients to a lesser extent than in healthy donors, the difference being statistically significant at a dose of 100 IU/ml of IFN-α (mean MxA units ± SD: 3.2 ± 1.4 vs. 4.8 ± 1.0, $P < 0.05$). Although monocytes are the main producers of the MxA protein [Towbin et al., 1992], these differences are not attributable to the cellular composition of PBMC, as cell subset counts were comparable in patients and healthy subjects. According to the viremic levels, PBMC from HBeAg-positive patients with a high viremia (HBV DNA positive by dot-blot hybridization) had a diminished MxA expression when compared with that of low viremic patients (HBV DNA positive only by PCR; Fig. 3), and with that of healthy donors, especially at doses of 10 and 100 IU/ml of IFN-α ($P < 0.05$). In addition, blood mononuclear cells from chronic HBV patients with abnormal ALT levels tended to express higher MxA amounts at low IFN-α concentrations than did those PBMC from patients with normal ALT levels, although the differences were not significant (data not shown).

The levels of the inducible enzyme 2-5A synthetase were also measured. Unstimulated PBMC had comparable, although slightly lower, values in patients than in healthy donors (Fig. 2B). Mononuclear cells from healthy donors showed a very strong, dose-dependent, induction of 2-5A synthetase activity in response to IFN-α ($P < 0.001$). This is in contrast to chronic hepatitis B patients, whose PBMC reached significantly lower induced levels of the bioactive enzyme ($P < 0.05$) at all the IFN-α doses tested (Fig. 2B). High viremic patients tended to have greater, although not significant 2-5A synthetase levels than did low viremic patients. MxA expression and 2-5A synthetase activity correlated significantly in healthy donors ($r = 0.82$, $P < 0.05$) and in chronic hepatitis B patients ($r = 0.52$, $P < 0.05$).

MxA responsiveness to type I IFN was observed similarly with all the types of recombinant or lymphoblastoid IFN-α tested: rhIFN-α2a, rhIFN-α2b, and IFN-αn1 (see Fig. 4, lane 1, for representative rhIFNα-2a results), as also occurred with IFN-β (Fig. 4, lane 2). Further investigation was carried out using other cytokines known to play a role in the establishment of different types of immune response. As shown in Fig. 4 (lanes 3 and 4, respectively), IL-2 and IL-12 elicited a weak induction of the MxA protein only slightly above the constitutive level of unstimulated PBMC. This effect was observed in 12 of 15 (80%) of the chronic HBV patients and in 4 of 6 (67%) of the healthy donors (P not significant) with all doses tested. As the levels of MxA expression in response to IL-2 and IL-12 were low in general, these did not differ significantly between patients and controls. Neither M-CSF, G-CSF, nor GM-CSF (Fig. 4, lanes 5–7, respectively) were able to stimulate MxA protein synthesis to amounts detectable above the unstimulated PBMC control.

DISCUSSION

The MxA protein is considered an antiviral mediator, and its role is being studied in several diseases of viral origin. Induction of the MxA protein is IFN dependent, and its expression therefore reflects the level of endogenous IFN production [Jakschies et al., 1990]. It has been suggested that the systemic production of IFN-α in chronic HBV patients is low in general [Nouri-Aria et al., 1991], and we have accordingly observed very low constitutive levels of MxA expression in PBMC iso-

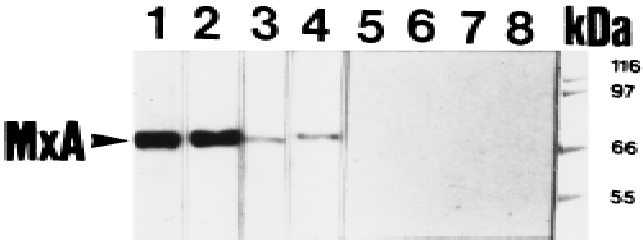

Fig. 4. Expression of the MxA protein in PBMC from chronic HBV patients. Immunoblot analysis of PBMC from patient no. 4 in Table I after stimulation with **lane 1:** 1,000 IU/ml of rhIFN-α2a; **lane 2:** 1,000 IU/ml of IFN-β; **lane 3:** 1,000 IU/ml of IL-2; **lane 4:** 100 ng/ml of IL-12; **lane 5:** 1,000 ng/ml of M-CSF; **lane 6:** 100 ng/ml of G-CSF; **lane 7:** 1,000 ng/ml of GM-CSF; **lane 8:** unstimulated.

lated from patients with a chronic HBV infection. We have subsequently analyzed MxA responsiveness to type I IFN and other cytokines. MxA responsiveness to IFN-α was diminished in chronic hepatitis B patients in comparison with healthy donors, and this effect was more marked in patients with high levels of HBV DNA in serum. These results indicate that HBV interferes with MxA induction, although the level at which this occurs and binding to the IFN receptor and/or the MxA transcription remain to be identified. The exact mechanism by which HBV affects the response to IFN treatment is unknown, although viral factors (i.e., HBV replication/gene expression) are apparently involved. The data presented suggest that continuous replication at high levels of HBV reduces the capacity of the patient's immune system cells to produce the MxA protein.

It is possible that chronic HBV infection leads to a downregulation of IFN receptors and thus to a poor IFN responsiveness. In this sense, another IFN-inducible gene, the enzyme 2-5A synthetase, also had markedly reduced levels in patients than in healthy donors in response to IFN. However, Nakajima et al. [1990] have shown that PBMC from chronic hepatitis B patients are able to respond to therapy with type I IFN, as is shown by a concomitant fall in the number of IFN receptors, together with a rise in the levels of the IFN-regulated 2-5A synthetase activity. A similar increase in 2-5A synthetase activity has recently been observed in HBV patients treated with GM-CSF alone or in combination with IFN-α [Martin et al., 1993]. These data suggest that PBMC are sensitive to the biological effects of IFN and that chronic HBV carriage may not affect IFN responsiveness. It may be that the reduced levels of MxA protein in PBMC from patients with a high virus replication are due to interaction of viral proteins with factors implicated in transcription through the MxA promoter. It has been recently reported that expression of the HBV capsid protein correlates negatively with expression of the MxA protein, but not with 2-5A synthetase, in Huh7 transfected cell cultures [Rosmorduc et al., 1996]. This finding is in agreement with the fact that replication at high levels of HBV genome would imply a high level of viral protein production and, in consequence, a reduction in transcription of the MxA gene [Rosmorduc et al., 1996]. The latter, and the observation that high viremic patients tended to have a greater 2-5A synthetase activity, is compatible with the hypothesis that MxA and 2-5A synthetase responsiveness to IFN-α are governed by different mechanisms, and with the narrower spectrum of the inducers of each gene transcription and expression [Staeheli, 1990], but this question remains to be elucidated in a future work.

It was found that IL-2 and IL-12 induce the MxA protein, but to a lesser extent than the type I IFN. It has been reported that IL-2 and IFN-gamma can induce the MxA protein via the production of IFN-α [Von Wussow et al., 1990]. To our knowledge, this is the first time that IL-12 has been shown to induce detectable levels of the MxA protein. It remains to be demonstrated whether IL-12, per se, induces MxA expression. Alternatively, this effect might be achieved through direct production of type I IFN or, indirectly, through a cascade effect, for it is well known that IL-12 is a strong activator of IFN-gamma synthesis [Trinchieri, 1994]. Some cytokines, like tumor necrosis factor-α, IL-1α, and IL-1β, require protein synthesis for MxA induction, and therefore they may act through induction of type I or type II IFN [Goetschy et al., 1989]. On the other hand, the lack of MxA induction by means of any of the CSF tested is in accordance with results reported by Von Wussow et al. [1990], as well as with our observations. Indeed, PBMC from HBV-infected patients receiving GM-CSF therapy produced small quantities of IFN-gamma, did not secrete spontaneously detectable amounts of IFN-α, and yet failed to respond to GM-CSF restimulation in vitro [Martin et al., 1994].

The results suggest that the persistence of HBV infection in chronic patients influences the production of the MxA protein. The results demonstrate that patients with low viremia and high disease activity usually have a better MxA inducibility in response to IFN-α stimulation in vitro. This finding is not surprising and is in accordance with recognized predictive factors for response to drug therapy in vivo [Perrillo, 1994]. These data provide an insight on which to base a better understanding of IFN-α effect on chronic HBV infection.

ACKNOWLEDGMENTS

We are grateful to Dr. D. Ando from Chiron-Cetus (Emeryville, CA) for providing us with M-CSF and IL-2 reagents and to Dr. P. Anchía from Schering-Plough (Spain) for providing us with GM-CSF.

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